STRUCTURE-ANTIOXIDATIVE ACTIVITY RELATIONSHIPS IN BENZYLISOQUINOLINE ALKALOIDS

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The antioxidative properties of the aporphines boldine, glaucine and apomorphine, and of the benzyltetrahydroisoquinolines (±)-coclaurine and (±)-norarmepavine were compared in the brain homogenate autoxidation model. The IC_{50} values found lay in the 16–20 μM range for the aporphines and were 131.7 μM, and 79.3 μM for coclaurine and norarmepavine, respectively. These results indicate that the antioxidative capacity (AC) of these compounds is related to the presence of the biphenyl system rather than phenol groups. The non-phenolic glaucine inhibited the 2,2'-azobis-(2-amidinopropane)(AAP)-induced inactivation of lysozyme with an IC_{50} value of 12 μM, while the corresponding values for the phenolic coclaurine and norarmepavine were 10 and 20 μM, respectively. *N*-Methylation of glaucine to its quaternary ammonium reduced its protective effect by two-thirds. This result suggests that a benzylic hydrogen neighbouring a nitrogen lone electron pair may be the key to the protective effect of non-phenolic aporphines.

KEY WORDS: antioxidants, benzylisoquinoline alkaloids, aporphines, SARs.

INTRODUCTION

The increasing acceptance of the role of reactive oxygen species and free radical reactions in a broad range of pathological processes has prompted the search for molecules with antioxidative properties as potentially useful therapeutic agents. Among the many synthetic and natural chemical structural types under study, the benzylisoquinoline alkaloids have recently emerged as a novel group of compounds with antioxidative activity [1–5]. Within this group of substances, our attention has focused, in particular, on the antioxidative properties of boldine [(*S*)-2,9-dihydroxy-1,10-dimethoxy-aporphine] (Fig. 1, 1), a major alkaloid present in the bark and leaves of boldo (*Peumus boldus* Mol., Monimiaceae) [6]. Boldine, at low micromolar concentrations, effectively prevents *in vitro* the auto-oxidation of rat brain homogenates, the chemically-induced (alkylperoxy radicals generated from the thermolysis of 2,2'-azobis-2-amidinopropane, AAP) peroxidation of red cell plasma membranes, the AAP-induced inactivation of the enzyme lysozyme [7], and the enzymatically catalysed and chemically (Fe^{2+}-ATP, Fe^{2+}-cysteine) induced peroxidative damage to rat liver microsomal membranes [5, 8]. These antioxidative actions of boldine are associated with a highly potent scavenging activity of the alkaloid against hydroxyl radicals (HO·), which are regarded amongst the most reactive oxygen metabolites generated by biological systems. Boldine is also effective in protecting *in vitro* microsomal membranes against the damage induced by *t*-butyl hydroperoxide and CCl_4, thus acting as a chain-breaking antioxidant [8]. The latter actions of boldine are likely to underlie its previously shown ability to protect hepatocytes *in vitro* against *t*-butyl hydroperoxide, and *in vivo* against CCl_4 [9].

Boldine is a diphenolic alkaloid in which each phenol function is somewhat shielded by an *ortho*-methoxyl group, a structural feature which is commonly associated with useful antioxidative properties. Not surprisingly, in the chemically induced (Fe^{2+}-cysteine) microsomal lipid peroxidation assay, Martínez *et al.* [5] found the catecholic norlaudanosoline and apomorphine (Fig. 1, 3) to be much more potent antioxidants than boldine; in the same assay the boldine isomer isoboldine, also containing two *ortho*-methoxyphenol moieties, and bulbocapnine, incorporating one such group, were found to inhibit peroxidation at somewhat lower concentrations than boldine. Nevertheless, the benzylisoquinoline alkaloid reticuline which, like

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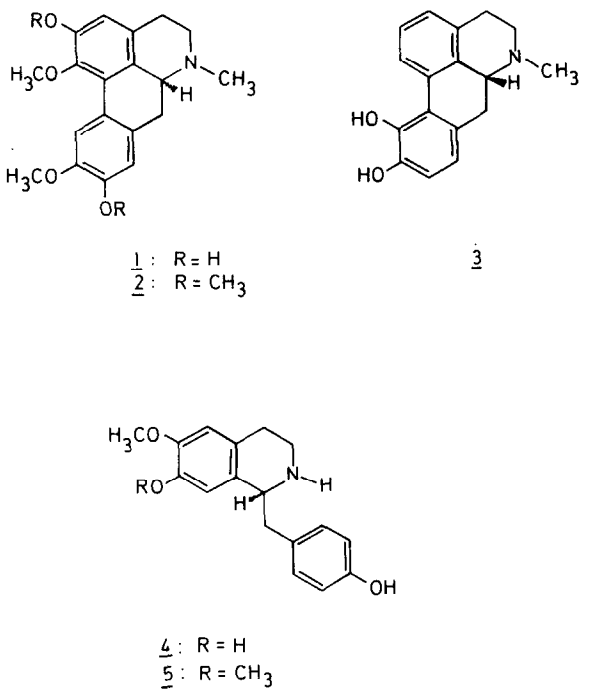

1: R = H
2: R = CH_3

3

4: R = H
5: R = CH_3

Fig. 1. Structures of the alkaloids used in this study: boldine (1), glaucine (2), apomorphine (3), (±)-coclaurine (4), (±)-norarmepavine (5).

boldine and isoboldine contains two *ortho*-methoxyphenol groups, exhibited little protective activity. On the other hand, the non-phenolic aporphine alkaloids glaucine (Fig. 1, 2) and anonaine were only slightly less potent than boldine as antioxidants [5]. These authors suggested that the protective effects of glaucine and anonaine might be related to 'the chemical properties of the aporphine nucleus, which can be readily oxidized to dehydroaporphine or oxoaporphine'. Such an explanation could be made extensive to the change in the behaviour of reticuline upon cyclization to isoboldine, but in neither case was any attempt made to discuss the mechanisms involved.

In the present work, in order to gain insight into the structural features underlying the antioxidative properties of aporphine alkaloids in general, and boldine in particular, we have compared the activity of several structurally related compounds. We have addressed the contribution of the phenolic moieties and that of the aporphine *vs* the benzylisoquinoline nucleus to the antioxidative capacities (ACs) of these substances in brain homogenate undergoing lipid peroxidation [10], and have also studied the influence of quaternization of the tertiary nitrogen atom of glaucine on its ability to protect lysozyme from inactivation by AAP-derived free radicals [11].

MATERIALS AND METHODS

Chemicals

(*S*)-Boldine (1) was isolated from the bark of *Peumus boldus* Mol. and used as the hydrochloride. (*S*)-Glaucine (2) was prepared by methylation of boldine with diazomethane and converted into its hydrobromide. *N*-Methylglaucinium iodide was prepared by reaction of glaucine with iodomethane. (±)-Coclaurine (4) was synthesized by a published method [12] and (±)-norarmepavine (5) was obtained by an analogous procedure. (*R*)-Apomorphine (3) hydrochloride and all other chemicals were purchased from Sigma (St. Louis, MO, USA), with the exception of AAP, which was from Polysciences (Warrington, PA, USA).

Pharmacology

Brain homogenate auto-oxidation. Male Sprague–Dawley rats (Instituto de Salud Pública, Santiago) weighing 200–250 g were kept on a standard pellet diet (Champion S.A., Santiago) *ad libitum*. The animals were anaesthesized with Nembutal (50 mg kg^{-1}, i.p.) and perfused with cold 0.15 M KCl through the portal vein to eliminate blood. Brains were removed, washed and stripped of meninges and blood clots using ice-cold phosphate–saline buffer solution (PSB) containing 140 mM NaCl and 40 mM potassium phosphate buffer pH 7.4 [13]. Tissue samples were homogenized (1:4) in PSB and centrifuged at 1000 *g* for 15 min at 4°C. The supernatants were immediately diluted (1:6) with PSB and kept on ice until use. Portions (3 ml) of the diluted brain homogenate were transferred to 15 ml glass vials for light emission studies [10]. Visible chemiluminescence was measured in a Beckman LS 6000TA liquid scintillation spectrometer, using single photon monitoring (Beckman Instruments, Fullerton, CA, USA) at 23–25°C. Light emission was recorded at 10 min interval for 1 h, either in the absence $(I_t)^\circ$ or presence (I_t) of different concentrations of the agents under study, and the respective ACs were calculated by

$$AC = 100 \times 1 - \frac{I_{60} - I_0}{(I_{60})^\circ - (I_0)^\circ}$$

where I corresponds to light emission at any given time t over that at time zero ($I=I_t/I_0$), with inferior indices indicating the time of measurement in min. Thiobarbituric acid-reactive substances (TBARS) were assayed as previously described [7].

Inactivation of lysozyme. Lysozyme activity was measured by following the loss of turbidity at 436 nm, when added to suspensions of lyophilized *Micrococcus lysodeikticus*, using the initial –dA/dt derivative (A=absorbance) [11]. Experiments were carried out at 45°C in 0.017 M NaCl containing 0.07 M potassium phosphate buffer pH 6.5 and 10 mM AAP, with or without (controls) different concentrations of the compounds under study.

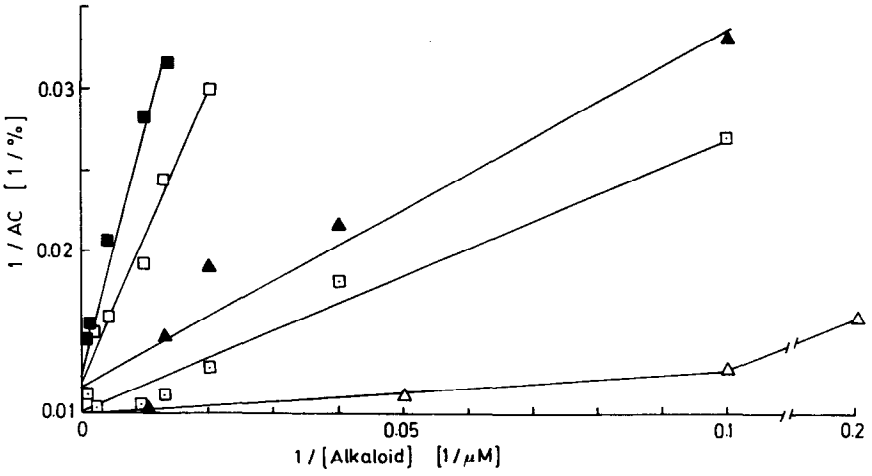

Fig. 2. Double-reciprocal plot of the antioxidative capacity (AC) of different benzylisoquinoline alkaloids as a function of their concentrations. The ACs were calculated as described in the Methods section from chemiluminescence data obtained in rat brain homogenates undergoing autoxidation. ⊡, boldine (19.6); □, norarmepavine (79.3); ■, coclaurine (131.7); △, apomorphine (3.4); ▲, glaucine (19.0). The numbers in parentheses adjacent to the alkaloid names represent the average inhibitory micromolar concentrations of the agents needed to decrease the peroxidation rate to 50% of that observed in their absence (IC_{50}), obtained by linear regression analysis.

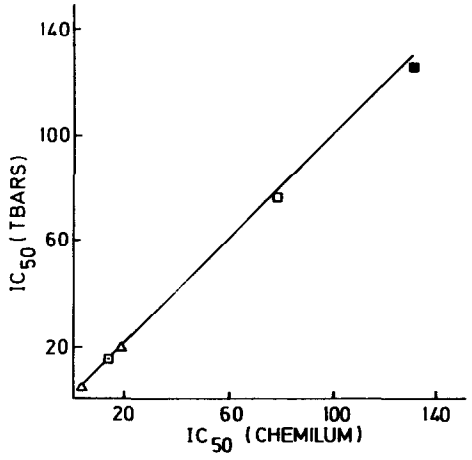

Fig. 3. Correlation between the antioxidative capacity (expressed as IC_{50}) of different benzylisoquinoline alkaloids measured (in brain homogenates) by chemiluminescence and the TBARS assay.

Statistics and expression of results. Results shown correspond to the average of three to five separate experiments (SEM values were omitted in the figures).

RESULTS

Rat brain homogenate auto-oxidation

The rate of rat brain homogenate auto-oxidation was evaluated from the light intensity emitted by the system during 60 min, either in the absence or presence of different alkaloids added within the concentration range of 5–892 μM. Under these conditions the alkaloids inhibited luminescence, allowing the calculation of both the AC values (by referring the data obtained at 60 min to those at time zero) and the respective IC_{50} values (by double reciprocal plot analysis of the AC of the compounds as a function of their concentrations) (Fig. 2). The data obtained indicate that, of the agents studied, apomorphine is the most efficient antioxidant (IC_{50}= 3.4 μM), followed by boldine and glaucine (IC_{50} of about 19 μM), norarmepavine (IC_{50}=79.3 μM), and coclaurine (IC_{50}=131.7 μM) (Fig. 2). The IC_{50} values of chemiluminescence were closely correlated (r= 0.98) with the results obtained using TBARS formation after addition of the antioxidant agents (Fig. 3).

Lysozyme inactivation induced by AAP

Incubation of lysozyme at 45°C with 10 μM AAP led to progressive enzyme inactivation as a function of time, expressed as the remaining activity at a given time [11]. The addition of compounds able to trap peroxyl radicals reduces the inactivation rate, but the profile of the remaining activity *vs* time plots depends on the antioxidant employed. Very efficient inhibitors whose reaction products do not present AC lead to well defined induction times. On the other hand, low efficiency inhibitors produce a decrease in the rate that is sustained for long incubation times. Antioxidants of intermediate efficiency or compounds whose oxidation products also present AC (i.e. albumin or boldine [7]) show more complex behaviours. Among the results obtained in the present study, those obtained employing glaucine are the most remarkable since this compound shows significant AC (see Fig. 4) in spite of its lack of phenol groups. For this compound, whose effect can be characterized as that of a mild antioxidant (i.e. there is no clearly defined induction time) it is possible to evaluate an

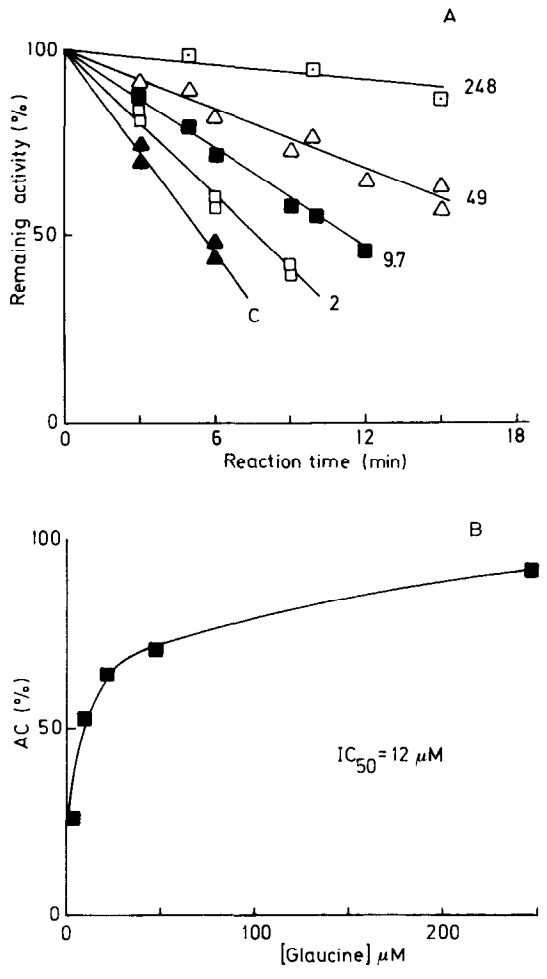

Fig. 4. Effect of glaucine on the inactivation of lysozyme initiated by AAP. (A) Time course of changes in enzyme activity studied in the absence (control, C) or presence of glaucine, at micromolar concentrations indicated by the numbers adjacent to the traces. (B) Antioxidative capacity (AC) of glaucine as a function of its concentration.

IC_{50} (12 μM) which is independent of the incubation time. For other antioxidants that show more complex behaviours (such as boldine), the inactivation rate changes with the elapsed time and it is difficult to obtain meaningful IC_{50} values. In order to allow a comparison of the relative protection afforded to lysozyme by these compounds, we have measured the ratio between the time required for the loss of 25% of the initial enzyme activity in the presence of 20 μM added antioxidant (t_{20}) and that required in its absence (t). IC_{50} values (for mild inhibitors) and t_{20}/t values are collected in Table I. Interestingly, the IC_{50} of glaucine is very close to that evaluated for boldine after the induction time [7], which may be considered as a measure of the AC of a boldine metabolite.

Table I
IC_{50} and t_{20}/t* values for the protection of lysozyme by some benzylisoquinoline alkaloids in the presence of AAP

Compound	*IC_{50} (mM)*	*t_{20}/t*
Coclaurine	10	3.0
Boldine	—	6
Norarmepavine	20	1.6
Glaucine	12	2.9
N-methylglaucinium	—	1.0

*Ratio between the time required for the loss of 25% of the initial enzyme activity in the presence of 20 μM added antioxidant (t_{20}) and that required in its absence (t).

DISCUSSION

In a recent publication [5], the (bis)catecholic benzyltetrahydroisoquinoline laudanosoline was found to inhibit Fe^{2+}/cysteine-induced lipid peroxidation in liver microsomal fractions with an IC_{50} of 6.8 μM, while its diphenolic but non-catechol analogue reticuline and the completely *O*-methylated laudanosine were unable to inhibit the same reaction at more than 100 μM. These results should be compared with the poor inhibition of spontaneous lipid peroxidation observed by us for the phenolic coclaurine (4) and norarmepavine (5), which suggests that high AC in benzyltetrahydroisoquinoline alkaloids *sensu stricto* is associated with the presence of catechol moiety.

The catecholic apomorphine (3) behaves as a potent antioxidant, both in the non-enzymatically-induced peroxidation of liver microsomes [5] and in brain homogenate autoxidation (Fig. 3), in line with results seen for other catechols. Nevertheless, non-catecholic aporphines also consistently exhibit good ACs with IC_{50} values generally below 30 μM. In view of the difference between this behaviour and that of simple benzyltetrahydroisoquinolines, it may be postulated that the superior ability of aporphines to trap free radicals is associated with increased spin delocalization of phenoxy radicals in the biphenyl system. The same occurs, however, even in the absence of hydroxyl groups as in the cases of the non-phenolic glaucine and anonaine, which is rather unexpected.

It has been pointed out [5] that aporphines are easily oxidized to dehydro- (actually 6a,7-didehydro) and oxo- (in fact 6-nor-7-oxo-4,5,6,6a-tetradehydro) aporphines, but nothing is known about the mechanism of these reactions. It seems reasonable to assume that, in non-phenolic aporphines, the benzylic C-6a–H bond may be the initial point of free radical attack. The resulting benzylic free radicals, by analogy with phenoxy radicals, would presumably be better stabilized in aporphines, as compared to their simple benzyltetrahydroisoquinoline-derived counterparts, by extended conjugation across the aporphine biphenyl system.

The choice of the C-6a radical as the key intermediate in the oxidation of nonphenolic aporphines, and thus in their antioxidative activity,

rather than the other possible (C-4, C-7) benzylic free radicals, is dictated by the presence of the neighbouring nitrogen lone pair. This moiety could be expected to contribute strongly to the stabilization of the C-6a radical by further extending electron delocalization beyond the biphenyl nucleus [14]. If this is so, however, sharing of the nitrogen lone pair should result in a loss of reactivity of protonated or quaternary non-phenolic aporphines towards free radicals. To test this hypothesis we prepared *N*-methylglaucinium iodide and compared it with boldine and glaucine in the lysozyme protection assay, as the quaternary salt was expected to be too lipid-insoluble for effectiveness in membrane-based assays. As anticipated, quaternization of glaucine to *N*-methylglaucinium resulted in virtually complete loss of lysozyme protective activity, supporting the idea that the additional stabilization provided by the nitrogen lone pair is essential in free radicals derived from non-phenolic aporphines.

In conclusion, AC in non-catecholic benzylisoquinoline alkaloids depends upon the presence of an extensively conjugated π electron system as in the aporphine nucleus, and of a hydrogen atom which may easily be removed to afford a free radical stabilized by spin delocalization over the π system. This labile hydrogen atom may be part of a phenol group, as has usually been assumed. Nevertheless, a benzylic hydrogen can apparently fulfill a similar role if it lies next to a basic nitrogen atom as in the aporphines, which could further delocalize the unpaired electron. According to this interpretation, sharing of the nitrogen lone pair with an additional electron acceptor (the quaternizing methyl group in the case of *N*-methylglaucinium) should destabilize the aporphine-derived free radical leading to the observed loss of AC.

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